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Metabolic responses to long-term food deprivation in subterranean and surface amphipods

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Abstract

A long-standing hypothesis in subterranean biology posits that organisms living in poor resource subsurface habitats can withstand long periods of bioenergetic shortages due to an innate reduced metabolic rate when compared to their epigean counterparts. However, previous studies have proposed that caves with ample energy resources may not evolve organisms with reduced metabolic rate. The equivocal nature of previous findings suggests that there is a need to compare food deprivation responses of subterranean and surface species in order to elucidate whether there are widespread adaptations to low energy systems in subterranean taxa. The purpose of the study was to examine patterns in basal metabolism and the effects of food deprivation in closely related subterranean- and epigean- amphipods, *Stygobromus pecki* and *Synurella* sp. from central and east Texas, USA, respectively. Basal metabolic rates (measured as O_2 consumption) differed between species, with *S. pecki* having substantially lower rates than *Synurella*. Individuals of both species were food deprived for a pre-determined time interval and changes in total body protein, lipids, and carbohydrates were measured throughout food deprivation experiments. *Stygobromus pecki* had larger initial energy stores than *Synurella* and were more conservative in the use of energetic reserves over a prolonged period of food deprivation. Thus, it appears that although *S. pecki* are currently found in shallow phreatic and spring opening environments, they have maintained more efficient metabolic adaptations to deal with prolonged periods of food deprivation.

Keywords

biochemical composition, hypogean, karst, metabolic rate, physiological adaptation, *Stygobromus pecki*, *Synurella*

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Introduction

Subterranean habitats are thought to be energy-limited ecosystems characterized by spatiotemporal patchiness of food resources (Culver et al. 1995; Juan et al. 2010). This is due to a lack of *in situ* autotrophic production and intermittent allochthonous (surface-generated organic matter) inputs of organic matter (OM) (Poulson 1964; Hüppop 1985; Venarsky et al. 2014; but see Hutchins et al. 2016). It is widely reported that hypogean (subterranean) organisms living in such low energy habitats have evolved a variety of behavioral, physiological, and metabolic adaptations that allow them to withstand long periods of resource shortage and show high starvation resistance (Hervant et al. 1997, 1999; Hervant and Renault 2002; Issartel et al. 2010). For example, some hypogean organisms have greater energetic reserves (e.g., greater glycogen, triglycerides, and protein content) and utilize these reserves at slower rates than related epigean taxa (Hervant et al. 1997, 1999; Hervant and Renault 2002; Mezek et al. 2010). Several studies have demonstrated that hypogean invertebrates exhibit physiological responses to prolonged food deprivation that differ from related epigean species. Hypogean amphipods and isopods respond to prolonged food deprivation in successive phases, with an immediate but slower rate of glycogen depletion, followed by utilization of proteins, and then use of lipids (Hervant et al. 1999; Hervant and Renault 2002). In contrast, epigean amphipods and isopods exhibit immediate, linear, and simultaneous declines in all energy reserve forms when exposed to periods of prolonged food deprivation (Hervant et al. 1999; Hervant and Renault 2002). However, it is unclear how conserved these metabolic responses to prolonged food deprivation is across hypogean fauna from different subterranean conditions.

Although organisms in many subterranean systems face low-energy conditions and rely heavily on infrequent inputs of surface-generated OM, there is growing recognition that some systems do not align with this paradigm. Some subterranean systems are relatively open and receive frequent and/or sustained inputs of allochthonous terrestrial OM (Culver and Poulson 1971; Schneider et al. 2011) or receive large inputs of high quality OM in the form of guano (Ferreira et al. 2000; Gnaspini 2005). In addition, some subterranean systems exhibit *in situ* chemoautotrophic production by microbial communities (Engel 2007; Porter et al. 2009), which can increase overall resource availability for organisms and lead to increased organismal diversity and greater food web complexity (Hutchins et al. 2016). It has been hypothesized that selection pressures to exhibit reduced metabolic rates or other adaptations to low energy conditions would be relaxed in these relatively energy-rich subterranean environments (Culver and Poulson 1971; Spicer 1998; Riesch et al. 2011). Thus, there is potential for subterranean obligate fauna to exhibit metabolism and physiological responses to prolonged food deprivation similar to those found in related epigean taxa, but there has been limited experimental examination of this hypothesis.

In addition to variation in resource availability among subterranean systems, many groundwater systems also exhibit vertical gradients in resource availability. Specifically, shallow phreatic habitats within aquifers, such as spring opening ecotones (i.e., transi-

tion zones between groundwater and surface–water habitats) are more likely to have greater access to terrestrial OM sources and therefore greater resource availability for faunal assemblages (Nair et al., in review). Spring ecotones often contain unique and diverse assemblages composed of surface, crenic (spring obligates) and hypogean taxa (Cantonati et al. 2012). Indeed, spring ecotone environments are considered "windows" to the subterranean world and present unique environments to investigate the ecology and evolution of groundwater organisms (Galassi et al. 2014). Although it has been hypothesized that subterranean fauna living in more energy-rich subterranean environments do not experience strong selection pressures to exhibit stygomorphic adaptations and should exhibit metabolic responses more akin to epigean species, this question has not been assessed for subterranean fauna that occupy shallow phreatic zones and spring opening ecotones.

The purpose of this study was to examine metabolic and food deprivation responses of a subterranean adapted organism that exists in a more energy-rich environment (i.e., shallow phreatic habitats and spring openings) in order to assess the hypothesis that subterranean organisms in more energy-rich environments experience relaxation of selective pressures on stygomorphic metabolic adaptations. These responses to prolonged food deprivation were then compared to food deprivation responses of a related surface species. Specifically, we examined the energy utilization (use of proteins, carbohydrates and lipids) and metabolic responses (O_2 consumption rates) to long-term food deprivation of two crangonyctid amphipods: the subterranean amphipod *Stygobromus pecki* and the largely epigean amphipod *Synurella* sp. *Stygobromus pecki* (Peck's cave amphipod) (Holsinger, 1967) is a federally endangered species endemic to two spring systems in the Edwards Aquifer of central Texas (i.e., Comal and Hueco springs) (United States Fish and Wildlife Service 1997; Gibson et al. 2008). *Stygobromus pecki* is found in shallow phreatic areas and at spring ecotones. It feeds largely on surface derived OM sources at its occurrence sites (Nair et al., in review), but is morphologically adapted for subterranean existence (e.g., eyeless, long appendages) (Holsinger 1967). In contrast, *Synurella* is widely distributed mostly epigean genus found in a variety of habitat types across the southeastern United States (including portions of Texas) and Europe (Holsinger 1977). The examination of metabolic responses of *S. pecki* to prolonged food deprivation and the comparison of these responses to a known epigean close relative presents a unique opportunity to examine the physiological responses and potential adaptations of a subterranean organism living in a more energy-rich environment.

Material and methods

Site description and organism collection

Live individuals of *S. pecki* were collected from spring openings at Comal Springs (29°43.0887'N, 98°7.8823'W). Comal Springs (city of New Braunfels, Comal County) is the largest spring complex in Texas and is located along the eastern edge of the

Edwards Plateau. The Comal Springs system discharges groundwater from the Edwards Aquifer from more than 400 spring openings and is the principle location for *S. pecki*. Live individuals of this species were collected from the immediate vicinity of spring openings by a combination of hand picking and sweeps of small aquarium nets. *Synurella* were collected using the same techniques from perennially flowing surface streams (30°35.8967'N, 95°7.71'W) near the city of Coldspring, Texas (San Jacinto County). Coldspring is located ~300 km to the northeast of Comal Springs. All known *Synurella* species in the southeastern United States are epigean (Holsinger 1977) and individuals collected at Coldspring are morphologically consistent with the epigean species *Synurella bifurca* (O.P. Hay, 1882). However, there is potentially substantial cryptic diversity within the genus that has yet to be resolved (J.R. Gibson, USFWS, personal communication). Thus, we hereafter have elected to refer to the species collected at Coldspring as *Synurella*. Live animals of both species were placed in high-quality coolers filled with site water and were brought back to the Freeman Aquatic Biology building at Texas State University (San Marcos, Texas).

Animals were acclimated to laboratory conditions in species-specific large plastic flow-through chambers with untreated Edwards Aquifer groundwater approximating the conditions found at collection sites [water temperature = 23 °C , dissolved oxygen (DO) concentration > 6 mg /L]. *Synurella* were exposed to a 12:12 light: dark cycle during housing, but *S. pecki* individuals were maintained in 24h darkness (Nowlin et al. 2015). Flow-through chambers contained pre-cleaned limestone river cobbles, polyester mesh, and well-conditioned conditioned wood and leaf material. Both species were fed *ad libitum* (typically weekly) with dense culture fish food (Pentair Dense Culture Food, F2A) prior to the start of experiments (Nowlin et al. 2015). Animals used in experiments were not differentiated based on sex due to the potential stress and mortality associated with sex determination. However, gravid females were excluded from the study in order to omit issues related to variation in biochemical composition. Individuals were held for a minimum of two weeks in the laboratory to acclimate to conditions before they were used for experiments.

Estimation of basal metabolic rates

We estimated basal metabolic rates of both species by measuring O_2 consumption. Oxygen consumption rates of well-fed individuals were estimated using Qubit systems OX1LP-30 DO cuvettes with Clark cell type polarographic oxygen sensor (Qubit Systems, Kingston, ON, Canada). The respirometric cuvette chamber had a small magnetic stirring bar positioned at the bottom of the chamber (but physically separated from the experimental animal) to continuously mix chamber water and ensure accurate O_2 concentrations in the chamber. The stirring rate was set to minimal speed in order to adequately mix chamber water but not induce stress on experimental animals. An individual test subject of either species was placed in a cuvette filled with 5mL of Edwards Aquifer water and was allowed to acclimate to the chamber for 30 minutes

prior to recording O_2 changes (in mg/L) of the chamber. After acclimation, DO concentration was recorded at 30-second intervals for 15 minutes. Cuvettes are externally jacketed with a water flow through system in order to maintain thermal stability at 23 °C. All experiments were carried out in a dark room. Per capita O_2 consumption rates were calculated by the dividing the change in DO concentration by 15 min. Mass-specific O_2 consumption rates (μmol O_2/g/h) were calculated by dividing O_2 consumption by wet weight of each individual (g). Oxygen consumption rates were estimated for n = 5 fed individuals of each species.

Food deprivation and metabolic responses

To assess biochemical changes and use of potential energy reserves in amphipods during extended periods of food deprivation, we experimentally examined change in whole-body metabolites of both species. Changes in metabolites during food deprivation was estimated at regular sampling intervals during food deprivation over 90 days for *S. pecki* (metabolites measured on days 0, 15, 30, 60, and 90) and over 30 days for *Synurella* (metabolites measured on days 0, 15, and 30). These species-specific food deprivation time periods were based on the literature and our own pilot experiments. Previous studies (Hervant et al. 1999; Hervant and Renault 2002) that compared metabolic responses to prolonged food deprivation in epigean and hypogean invertebrates found that hypogean species could withstand longer periods of starvation. Our pilot study that assessed mortality during an extended food deprivation period in the laboratory for both *S. pecki* and *Synurella* and found that *S. pecki* exhibited could withstand a substantially longer food deprivation period of time before death (~120 days) than *Synurella* (~45 days).

Prior the start of food deprivation experiments, animals were acclimated to laboratory conditions, maintained, and fed as above for ~1 month. At the start of experiments, individuals of each species were separated into two treatments: fed (receiving weekly dense culture fish food pellets) and unfed (food deprived). For both species, each treatment contained n = 75 individuals. Individual animals were housed in 4 cm long and 1.91 cm diameter PVC flow-through holding chambers. Amphipods were segregated from each other during experiments in order to prevent cannibalism (Nowlin et al. 2015). Edward Aquifer groundwater was continuously fed through holding chambers to ensure volume was replaced every 2–3 minutes. On each sampling date, randomly selected individual amphipods from each of the four treatment groups (*Synurella* fed or food deprived and *S. pecki* fed or food deprived) were removed for metabolite analysis. Because of sample mass requirements for biochemical analysis, three individuals of each treatment were pooled prior to analysis (a pilot study was conducted and determined that n = 3 individuals were required to exceed metabolite analytical detection limits). Thus, on each sampling date each treatment had n = 5 analytical replicates (each replicate consisting of three randomly selected individuals). Animals were checked weekly for mortality; animals were not replaced in cases of mortality.

Metabolite analysis

Whole-body metabolites were estimated on each sampling date (Day 0, 15, 30, 60 and 90 days for *S. pecki* and day 0, 15, and 30 for *Synurella*). In order to minimize the inclusion of food materials in the guts of animals in biochemical analyses, animals were removed from chambers prior to weekly feedings; no leftover food was observed in holding chambers when animals were removed for analysis. In addition, once animals were removed, they were held for ~2 hours to clear gut contents and then frozen at - 80 °C in clean 2 mL microcentrifuge tubes. Before metabolites were assayed, animals were thawed and wet weight of each individual was determined (mg). For each sample (composed of n = 3 individuals), tissue was homogenized and one-third of the tissue was further homogenized in phosphate buffer solution (pH = 7.4) for protein and total carbohydrate analysis. Proteins were analyzed using a Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific) and total carbohydrates were analyzed using a total carbohydrate assay kit (Cell Biolabs, STA-682). Remaining tissue for each sample was homogenized in 2:1 chloroform-methanol (v/v) and lipids were extracted using a modified procedure by Folch et al. (1957), and quantified (unsaturated fatty acids only) using a lipid quantification kit (Cell Biolabs, STA-613). All assays were performed using a spectrophotometer at 25 °C.

Data analysis

For analysis of basal metabolic rates, difference in mass-specific O_2 consumption rates between species was assessed using one-way ANOVA. For the food deprivation experiment, body composition (protein, carbohydrate, and lipid content) between species was compared on Day 0 (immediately prior to the start of food deprivation) using one-way ANOVA. The effect of food deprivation on body composition within each species was then assessed by comparing treatments (fed *versus* unfed) with repeated measures ANOVA, which provides the treatment effect (fed *versus* unfed), time effect (Days 15 and Day 30 dates for *Synurella*, Days, 15, 30, 60, and 90 for *S. pecki*), and the treatment x time interaction. Prior to analyses, data were examined for normality, homoscedasticity, and sphericity (for the repeated measures ANOVA). If data did not meet assumptions, they were either *ln*- or square root-transformed. Significance for all tests was inferred at $P \leq 0.05$ and analyses were performed in R (version 3.5.0, R Core Team 2018).

Results

O_2 consumption rates

Basal metabolic rates differed between *S. pecki* and *Synurella* ($F_{1, 8} = 15.99$, $P < 0.004$; Fig. 1). Mass-specific O_2 consumption rates of *S. pecki* (3.3 ± 0.9 μmol /g/h) were significantly lower (by an order of magnitude) than those of *Synurella* (32.5 ± 7.23 μmol/g/h).

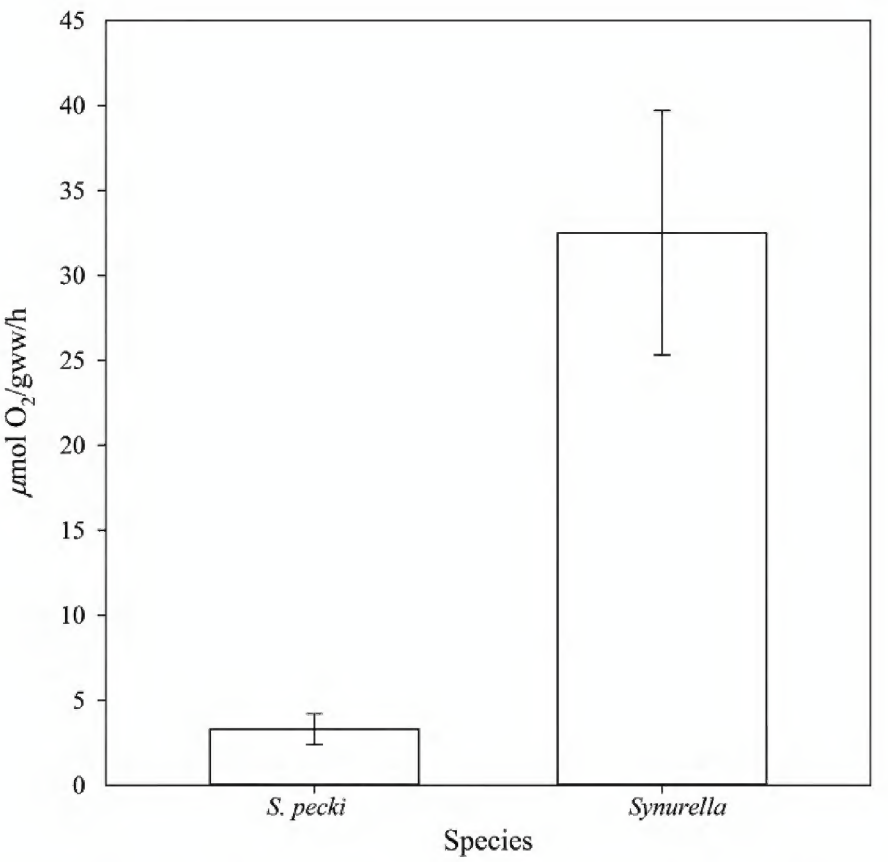

Figure 1. Oxygen consumption in darkness for *Stygobromus pecki* and *Synurella* at 23 °C. Values are means ± Standard Error Means (SEM) for n = 5 animals.

Effects of food deprivation on metabolite content

Prior to the start of experiments (Day 0), the content of some metabolites differed between the two species (Fig. 2A–C). Total carbohydrate content was higher in *S. pecki* ($F_{1, 8}$ = 43.83, P < 0.001). However, protein content did not differ between species ($F_{1, 8}$ = 1.46, P = 0.261), but lipid content was higher in *Synurella* ($F_{1, 8}$ = 7.40, P = 0.026).

During the period of food deprivation, total carbohydrate content of *S. pecki* did not differ between fed and unfed treatments ($F_{1, 4}$ = 0.356, P = 0.583; Fig 2A). However, in both treatments, total carbohydrate content of animals declined with time ($F_{3, 12}$ = 19.89, P < 0.001), but the rate of carbohydrate reduction did not vary with treatment (Time × Treatment: $F_{3, 12}$ = 0.456, P = 0.718). In contrast, food deprivation in *Synurella* led to an immediate reduction in total carbohydrates ($F_{1, 8}$ = 17.44, P = 0.003; Fig. 2A). Total carbohydrate content in *Synurella* varied temporally (Time: $F_{1, 8}$ = 15.61, P = 0.004), but this temporal variation was dependent upon whether *Synurella* were fed or deprived of food (Time x Treatment: $F_{1, 8}$ = 10.11, P = 0.013).

Protein content of *S. pecki* differed between fed and unfed treatments, with fed animals having higher protein content ($F_{1, 8}$ = 19.28, P = 0.023; Fig. 2B). Protein content varied through time in both treatments ($F_{3, 24}$ = 3.17, P = 0.043), but there was no interdependence between treatment and time ($F_{3, 24}$ = 0.316, P = 0.813). Food deprivation of *Synurella* led to an immediate reduction in protein content ($F_{1, 8}$ = 8.07, P = 0.022; Fig. 2B), but content did not vary temporally after 15 days of food deprivation (Time: $F_{1, 8}$ = 5.17, P = 0.0517; Time × Treatment: $F_{1, 8}$ = 2.55, P = 0.149).

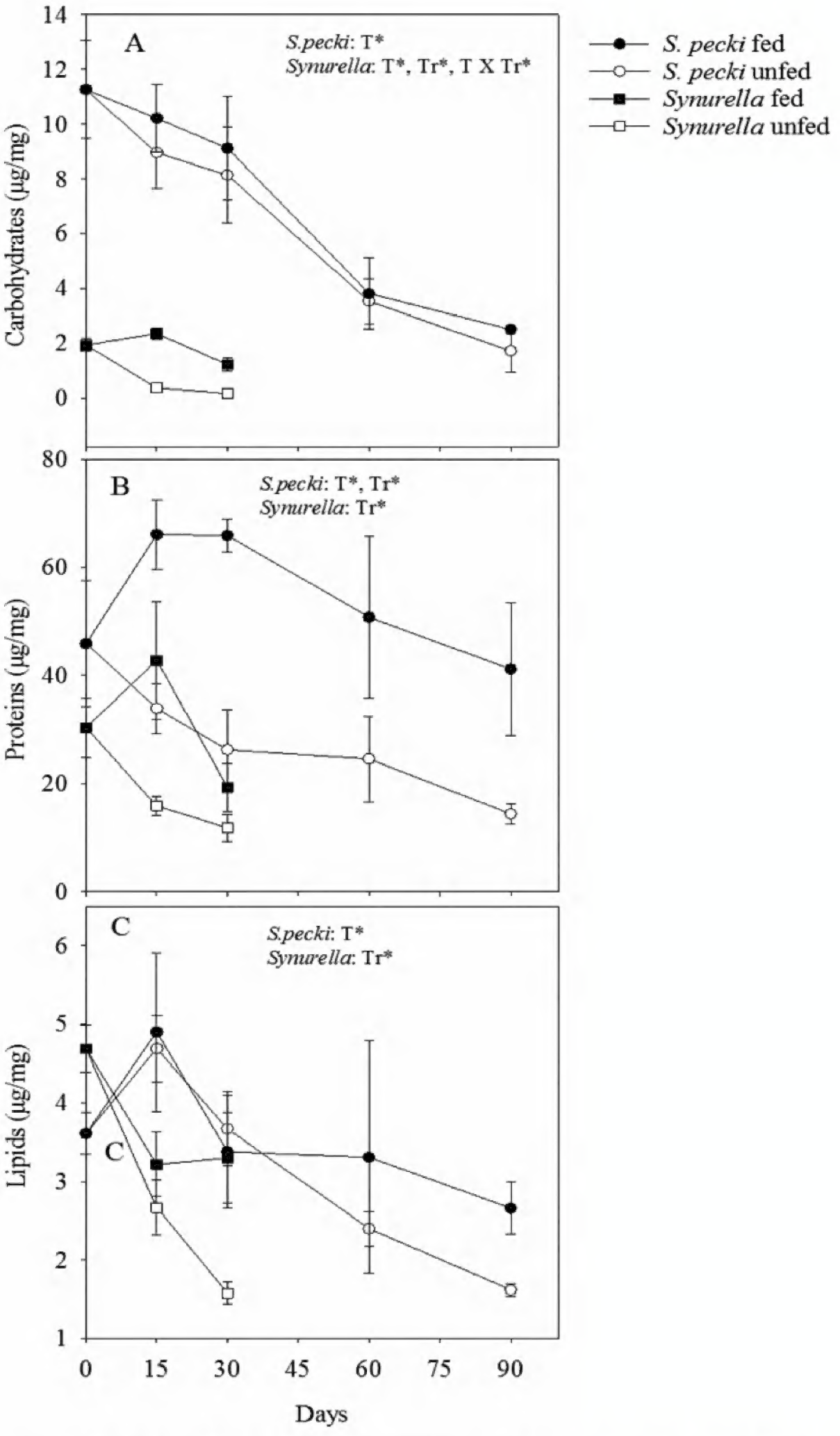

Figure 2. Changes in the levels of body metabolites in *Stygobromus pecki* and *Synurella* sp. **A** Carbohydrates **B** proteins **C** lipids concentrations during long-term food deprivation at 23 °C in darkness. Values are means ± SEM for n = 5 replicates. (*) indicates significance at $P < 0.05$ for the main effects of Treatment, Time and the Time × Treatment interaction.

Lipid content of *S. pecki* did not differ between treatments during the food deprivation period ($F_{1, 8}$ = 2.51, P = 0.152; Fig. 2C). Lipid content of *S. pecki* varied through time in both treatments ($F_{3, 24}$ = 4.43, P = 0.01), but this variation did not depend upon treatment (Time × Treatment: $F_{3, 24}$ = 0.481, P = 0.698). For *Synurella*, lipid content was higher in the fed treatment during the starvation period ($F_{1, 8}$ = 15.66, P = 0.004; Fig. 2C). However, once the period of food deprivation started, *Synurella* lipid content did not vary temporally (Time: $F_{1, 8}$ = 2.95, P = 0.124; Time × Treatment: $F_{1, 8}$ = 2.91, P = 0.127).

Over the entire experimental period, mortality of *Synurella* was 24% for fed and 38% for unfed treatments. *S. pecki* mortality was 18% for fed and 37% for unfed treatments.

Discussion

Capacity to withstand periods of low to no food supply depends on the presence of endogenous reserves and metabolic responses that ensure efficient utilization of stored metabolites (Hervant et al. 1997, 1999, 2001; Fuglei et al. 2000; Issartel et al. 2010). Previous studies have reported species-specific differences in storage and utilization of metabolites in epigean and hypogean crustaceans during periods of food deprivation (Hervant et al. 1999, 2001; Hervant and Renault 2002; Sacristán et al. 2016). In the current study, related hypogean and epigean amphipods exhibited markedly different physiological strategies to deal with food deprivation. Lower overall metabolic rates observed in *S. pecki* (in comparison to *Synurella*) are in accordance with studies that have found that hypogean organisms have lower metabolic activity when compared to surface relatives (Hervant et al. 1997, 1998; Spicer 1998; Hervant and Renault 2002; Simčič et al. 2005; Mezek et al. 2010). *Stygobromus pecki* used in experiments were substantially larger than *Synurella* (*S. pecki* mean wet mass = 13.4 mg, *Synurella* mean wet mass = 7.1 mg) and metabolic scaling indicates that mass-specific metabolic rates decline with increasing body size (Brown and Sibly 2012). However, when we express O_2 consumption rates on a per capita basis (μmol O_2 consumed/h per individual), *Synurella* still exhibits a substantially greater basal metabolic rate than *S. pecki* (~5× greater), further indicating that *S. pecki* has lower basal metabolic rates even when body size differences are considered. In addition, presence of greater total carbohydrate stores in *S. pecki* (relative to *Synurella*) on Day 0 prior to start of food deprivation is in line with previous studies that have compared metabolic responses of epigean and hypogean taxa to food deprivation (Hervant et al. 1999, 2001; Hervant and Renault 2002). Presence of greater energy stores in hypogean species is thought to be indicative of adaptation to low energy subterranean conditions so that individuals can continuously fuel metabolic needs for longer periods of low food availability. Thus, results from this study indicate that despite the shallow subterranean existence of *S. pecki* (and its presumed greater access to food resources), basal metabolism and initial energy reserves in this species seem to follow the paradigm that subterranean species have relatively depressed metabolic rates and different energy reserve concentrations when compared to epigean fauna.

In the present study, lipid reserves were substantially higher in *Synurella*, when compared to *S. pecki*. In contrast, Hervant et al. (1999) found that that the subterranean amphipods, *Niphargus virei* and *N. rhenorhodanensis* had higher stored lipids and carbohydrates (as glycogen) than surface amphipods (*Gammarus fossarum*). Contrasting results from Hervant et al. (1999) and this study could be due to differences in species-specific adaptations to food limitation resulting from different ways of life and metabolic needs (Danielopol and Rouch 1991). In addition, species-specific differences in storage and utilization of body reserves may occur due to differences in thermal adaptation, type and quality of food consumed, feeding history and life-cycle strategy, as well as their biosynthesis from either dietary fatty acids or dietary proteins, carbohydrates or lipids (Lahdes et al. 2010; Pond 2012). The two species which Hervant et al. (1999) studied (*N. virei* and *N. rhenorhodanensis*) are typically found in deep phreatic habitats, whereas *S. pecki* is a shallow phreatic zone specialist. Thus, it is possible that differences between the findings of this study and that of Hervant et al. (1999) could come from the evolutionary history, specific habitat associations, and access to food resources of the specific fauna used in experiments.

Although *S. pecki* did not have higher lipid stores than *Synurella*, the present study found differences between epigean and hypogean species in terms of utilization and depletion of various energy reserves during starvation. Starvation leads to changes in the body composition (Gibert and Mathieu 1980; Barclay et al. 1983), and the relative importance of different metabolite reserves depends on the duration of starvation as well as species-specific differences in metabolism and regulation (Hervant et al. 1999; Caruso et al. 2008). During starvation, crustaceans meet energy demands by regulating enzymatic activities to access energy reserves (i.e., hydrolysis of proteins to amino acids, glycogen to glucose, and triglycerides to free fatty acids), while ensuring cell integrity (Sánchez-Paz et al. 2006). In the present study, *Synurella* demonstrated a monophasic response to food deprivation, characterized by an immediate linear decrease in all energy reserves, including lipids. In contrast, the only metabolite that differed between fed and unfed treatments during starvation of *S. pecki* were proteins. Thus, it appears that *S. pecki* accessed proteins as an energy reserve during the 90-day starvation period. Hervant et al. (1999) observed that glycogen stores were first utilized by food deprived hypogean amphipods (*Niphargus* sp.) and that protein reserves were sparingly utilized after 30 days of food deprivation. Thus, initial utilization of protein energy reserves during periods of starvation may not be a cosmopolitan feature of hypogean amphipod metabolism, but further comparative studies are required.

Our study found that the use of bulk energy reserves differed between the two crangonyctid species we examined. *Synurella* exhibited monophasic declines in all bulk energy reserves during food deprivation, whereas *S. pecki* only exhibited differences in protein content in fed and food deprived animals. However, it is likely that our analysis of bulk energy reserves (i.e., total lipids and carbohydrates) may have obscured differences between the two study species in the utilization of specific energy reserve constituents during periods of food deprivation. Within crustaceans, neutral lipids (mainly triglycerides) are preferentially catabolized during food deprivation, but po-

lar lipids (i.e., phospholipids and cholesterol) are conserved because of they serve as structural components of cell membranes (Hervant et al. 1999). Similarly, crustaceans access carbohydrate reserves stored mainly as glycogen when they are food deprived (Hervant et al. 1999; Hervant and Renault 2002; Sánchez-Paz et al. 2006). Given the differential use of energy reserve constituents (i.e., triglycerides versus phospholipids, glycogen versus glucose) during food deprivation periods, future studies should attempt to examine these constituents in order to better elucidate patterns of energy use within and among our study species.

In this study, carbohydrate reserves in *S. pecki* were depleted in both fed and unfed populations in the lab throughout the experimental period. *Stygobromus pecki* in the fed treatment were supplied food at similar rates to other *S. pecki* individuals we have maintained in the laboratory and it appeared as though they were consuming the added food items (P. Nair, personal observation). The reason or mechanisms for carbohydrate depletion in fed *S. pecki* in the current experiments is not known, but it may be due to stress associated with being held in captivity in a for a relatively long period of time. Some hypogean species can be sensitive to being held in captivity for extended time periods; Gibert and Mathieu (1980) found that individuals of the hypogean amphipod *N. rhenorhodanensis* that were fed and kept under conditions close to their natural environment in the lab showed significant reduction in carbohydrates reserves within a month of being held in captivity. These authors hypothesized that this was due to captive stress, but this hypothesis requires further study. The USFWS maintains captive populations of *S. pecki* at the San Marcos Aquatic Resource Center facility (SMARC; San Marcos, Texas), but it is unclear if these individuals similarly experience carbohydrate reduction or any stress associated with being held in a captive setting. Clearly, there is a need to further explore these results from an applied and captive breeding perspective as it applies to *S. pecki* and its endangered conservation status.

Conclusions

Our study shows that *S. pecki* has lower energetic requirements (i.e., basal metabolic rates), greater total carbohydrate reserves, and lower rates of lipid use during starvation when compared to a surface relative. Cumulatively, these findings suggest that *S. pecki* maintains a stygomorphic metabolic strategy for survival in environments with low or sporadic food availability, despite its occurrence in shallow phreatic and spring opening environments. *S. pecki* is closely related to amphipod species which occur in deeper phreatic environments (Ethridge et al. 2013) and it is hypothesized that ancestors of these taxa invaded freshwater subterranean systems during the late Cretaceous or early Cenozoic (Holsinger 1967). Thus, present-day metabolic adaptations to persistence in a deeper phreatic low-energy environments observed in *S. pecki* is consistent with their longer-term evolutionary history, rather than their current distribution in and around surface spring opening ecotones. *Stygobromus pecki* serves as the top invertebrate predator in spring opening ecotones, feeding on surface invertebrates, including the epigean

hyalellid amphipod *Hyalella azteca* (Nair et al., in review) and *S. pecki* has the ability to detect and actively avoid light, presumably to avoid predation (Nowlin et al. 2015; Worsham et al., in preparation). The juxtaposition of some surface-adapted traits (i.e., surface foraging, light avoidance) and subterranean metabolic characteristics in *S. pecki* suggests that metabolic traits are not easily modified even though *S. pecki* now lives in shallow groundwater – surface water interfaces. However, future studies focused on comparing metabolic requirements and response of *S. pecki* with their deep phreatic relatives are needed to further evaluate the hypothesis that *S. pecki* exhibits deeper phreatic metabolic characteristics despite its current shallow phreatic distribution.

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